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INDUCTION OF HYPOZINCEMIA AND HEPATIC METALLOTHIONEIN SYNTHESIS IN
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Recent evidence indicates that hypersensitivity reactions, produced in rats by the administration of a protein antigen, alters plasma zinc and iron homeostasis by depressing concentrations of these trace minerals. Studies were performed to determine if hypozincemia occurs as a consequence of redistribution of zinc from plasma to liver by a mechanism involving enhanced hepatic metallothionein (MT) synthesis. MT, a high cystein-containing cytoplasmic protein, possesses a high affinity for zinc and other heavy metals and has been implicated in zinc

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homeostasis. Antigen challenge (0.5 mg BSA) in rats previously sensitized with either 5 or 10 mg BSA produced a significant decrease in plasma zinc and iron concentrations within 7 hr in an apparent dose-dependent manner. Plasma zinc depression was accompanied by an increase in hepatic MT content as well as MT-associated total Zn and ^{65}Zn used to pulse-label the metalloprotein. The depression in plasma zinc, but not iron, and the enhanced synthesis of MT was significantly reduced by prior treatment of rats with actinomycin D. This finding suggests a requirement for new mRNA synthesis for zinc, but not iron alterations during hypersensitivity reactions. Results support the concept that induction of hepatic MT may be a common mechanism involved in altered plasma zinc homeostasis regardless of the initiating pathophysiologic condition.

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(6) Induction of Hypozincemia and Hepatic Metallothionein Synthesis in Hypersensitivity Reactions[1]

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Running title: METALLOTHIONEINS AND HYPERSENSITIVITY

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[1] In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" as promulgated by the Committee on the Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

Antigen challenge of rats and rabbits previously sensitized to the same protein antigen has been demonstrated by Kampschmidt and Pulliam (1) to induce depressions in plasma zinc and iron concentrations. However, these authors did not determine if the depressions of plasma trace metals which occur during hypersensitivity reactions are the result of redistribution of the metals from plasma to liver. Such redistribution has been documented in infectious disease (2), inflammation (3), and endotoxemia (4).

Our recent investigations into the mechanisms involved in the hypozincemia of bacterial infections (5), inflammation (6) and endotoxemia (7) have shown that induction of hepatic metallothioneins (MT) in rats is an integral part of the redistribution of zinc from plasma to liver which occurs in these conditions. Due to their high number of half-cystinyl residues, MT possesses a rather unique ability to sequester various bivalent metals such as zinc, cadmium, and mercury as mercaptide complexes (8). Considerable evidence has accumulated which indicates that the synthesis of hepatic MT is inducible and mediated at the transcriptional level (9). These proteins are currently under intensive investigation in an attempt to delineate their biological function(s).

The present study was performed to determine whether zinc accumulates in the liver during hypersensitivity reactions and if so to what extent this is dependent on the induction of hepatic MT synthesis. In addition, the influence of hypersensitivity reactions on the plasma concentrations of iron was simultaneously assessed.

Materials and methods

Male Fisher Dunning rats weighing 234 ± 3 g (mean ± SEM) were used after a minimum of 1 week acclimation under controlled environmental conditions as previously described (5). Food (Wayne Lab-Blox, Allied Mills) and water were provided ad libitum.

Hypersensitivity reactions were produced essentially by the procedure described for rats by Kampschmidt and Pulliam (1) except that in one series of experiments a larger sensitizing dose of protein antigen was used. Briefly, a solution of bovine serum albumin (BSA, Sigma) in sterile water was prepared to contain 100 mg/ml and emulsified with an equal volume of Freund's complete adjuvant (Difco). A 0.1 ml aliquot of this suspension was injected sc into either one or both hind foot pads to achieve sensitizing doses of 5 and 10 mg respectively. Rats were challenged 14 days later by ip administration of 0.5 mg BSA dissolved in sterile water. Control groups included sensitized unchallenged as well as unsensitized challenged rats.

Blood samples were collected from the pleural cavity 7 hr after the time of challenge and processed as previously described (5) for the determination of plasma zinc (10) and iron (11) concentrations. Livers were extirpated immediately after exsanguination and processed for the isolation of MT by the combined procedures described by Sobocinski et al. (5). These procedures include preparation of liver homegenates, high-speed centrifugation, heat-denaturation, acetone fractionation, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Metallothioneins were labelled in vivo (5) with a pulse dose of ^{65}Zn, 10 μCi/100 g body weight (carrier-free ^{65}Zn, New England Nuclear)

administered ip immediately after the time of challenge. ^{65}Zn-activity in hepatic MT fractions was determined as previously described and expressed as cpm/ml heated cytosol (5). Protein content of MT fractions was not determined since neither biuret nor Lowry procedures yield valid results (12). In some experiments relative hepatic MT content between various experimental groups was estimated by gravimetric analyses of peak areas obtained by densitometry (Corning 740 system) of stained gels after SDS-PAGE (5). Zinc content of MT fractions was determined by atomic absorption spectrophotometry (10).

In certain experiments, actinomycin D (Calbiochem) pretreatment was used to inhibit MT synthesis (13) and was administered sc (0.08 mg/100 g body weight) to sensitized rats 1 hr prior to challenge. Control rats received an equivalent volume of propylene glycol used to solubilize the actinomycin D (5).

Differences between group means were evaluated for significance by one-way analysis of variance. A P value ≤ 0.001 was considered significant.

Results

The effect of sensitization with or without subsequent challenge and challenge without sensitization on plasma concentrations of zinc and iron is shown in Table I. Challenge of rats sensitized with 5 mg BSA produced a significant decrease in zinc and iron concentrations when compared to values obtained in sensitized unchallenged rats or those animals which received only the standard challenge dose of 0.5 mg BSA. In addition to the effect on plasma zinc concentration in sensitized rats, challenge induced a significant increase in the Zn and ^{65}Zn content of the hepatic MT fraction when compared to controls. The effect of sensitization or

challenge alone on the zinc content of the MT fraction was similar.

Typical SDS-PAGE separation of the two MT variants, forms A and B (5), isolated from livers of rats sensitized with 10 mg BSA and challenged 14 days later with 0.5 mg antigen is shown in Fig. 1. Normally, only a very small amount of MT is detectable in livers of control animals. The behavior of these MT forms on SDS-PAGE was identical to that previously described by us for MT isolated from livers of infected rats (5).

In a second series of experiments, a 10 mg sensitizing dose of BSA and actinomycin D pretreatment was used to demonstrate the inducibility of MT during hypersensitivity reactions. The effect of sensitization with the higher dose of BSA and subsequent challenge on plasma zinc and iron concentrations is shown in Table II. In contrast with the data obtained with a 5-mg sensitizing dose (Table I), challenge with the same amount of BSA produced a greater decrease in plasma zinc and iron. Other data presented in Table II demonstrate that sensitization with 10 mg antigen rather than 5 mg (Table I) increases the amount of MT-associated zinc (MAZ) by a factor of 2.4.

Pretreatment of sensitized rats with actinomycin D prior to challenge significantly reduced MAZ as well as MT content when compared to drug controls and prevented the hypozincemia associated with challenge in these rats (Table II). In contrast, actinomycin D pretreatment had no apparent effect on the depression of plasma iron induced by hypersensitivity reactions.

Discussion

Results obtained in this study have added to our previously published evidence (5-7) documenting the apparent fundamental importance of hepatic MT in altered zinc homeostasis occurring in disease states and after the

administration of phlogistic agents. Others (14) have recently reported an increase in hepatic MT content during stresses which included cold exposure and strenuous exercise. The induction of hepatic MT is also known to occur after the administration of various heavy metal salts (15) and during food restriction (13). It is unknown whether a common stimulus or several different stimuli exist to explain MT induction in seemingly unrelated stresses.

To explain the hypozincemia and hypoferremia associated with hypersensitivity reactions, Kampschmidt and Pulliam (1) proposed that leukocytic endogenous mediator (LEM) is released from phagocytic cells after stimulation by lymphocytic substance(s). When administered to rats, LEM has been shown to induce numerous host metabolic responses which include depressions in plasma zinc and iron concentrations (16). A similar concept has been previously used to explain the etiology of fever during delayed hypersensitivity (17). Evidence presented by Atkins and Francis (17) suggests that lymphocytes produce lymphokine-like substances which stimulate phagocytic cells to produce endogenous pyrogen (EP), the mediator of febrile response. Controversial evidence exists, however, concerning the differentiation of LEM and EP (18, 19). Although the model used in the present studies was originally described as "delayed" hypersensitivity (1) it has not been established, to the best of our knowledge, to what extent the measured responses to antigen challenge involve antibody and/or cell-mediated immune responses. The use of an ip injection and the rapid responses of serum zinc and hepatic MT suggest that the acute formation of antigen-antibody complexes constituted the triggering stimulus. In the absence of conclusive proof, the involvement of LEM in the hypozincemia and hypoferremia of hypersensitivity reactions

remains highly speculative. Although we have shown that LEM administration to rats can induce MT-like hepatic zinc-binding proteins (7), we have been unable to demonstrate a direct effect of LEM on the perfused rat liver with respect to MT induction (unpublished observations).

In contrast to the proposed role of LEM as a mediator of inflammatory hypoferremia (20, 21), recent data presented by Van Snick *et al*. (22) indicates that leukocytic lactoferrin mediates plasma iron depression during inflammatory stress. This evidence together with that obtained in this study concerning MT and plasma zinc depression suggests a key role for these two distinct metalloproteins in modulating plasma zinc and iron homeostasis.

Our observation that actinomycin D pretreatment can significantly reduce hepatic MT content is compatible with the concept that new mRNA is required for enhanced MT synthesis (9, 23, 24). Although it is also possible that the drug interferes with production of endogenous mediator(s) such as LEM, no conclusive evidence is available to indicate a mRNA requirement for the production of potential mediator(s) involved in plasma zinc depression. The lack of complete inhibition of MT synthesis by actinomycin D may be due, in part, to the presence of endogenous mRNA synthesized, but not translated, during the induction of the hypersensitive state. The dose of actinomycin D used in the present experiments has been previously shown to inhibit MT synthesis when administered as early as 30 min prior to induction by cadmium (24). The apparent lack of a stoichiometric relationship between changes in plasma zinc concentration and hepatic MT content in drug-treated rats may be attributed to the effect of actinomycin D on zinc clearance from blood (13).

It appears reasonable to conclude that evidence obtained in this study and others (5-7, 14) indicates that MT induction is a common feature of altered plasma zinc homeostasis. To fully understand the pivotal role of MT in zinc metabolism, the event(s) leading to the initiation of mRNA synthesis must be further defined.

Summary

Recent evidence indicates that hypersensitivity reactions, produced in rats by the administration of a protein antigen, alters plasma zinc and iron homeostasis by depressing concentrations of these trace minerals. Studies were performed to determine if hypozincemia occurs as a consequence of redistribution of zinc from plasma to liver by a mechanism involving enhanced hepatic metallothionein (MT) synthesis. MT, a high cystein-containing cytoplasmic protein, possesses a high affinity for zinc and other heavy metals and has been implicated in zinc homeostasis.

Antigen challenge (0.5 mg BSA) in rats previously sensitized with either 5 or 10 mg BSA produced a significant decrease in plasma zinc and iron concentrations within 7 hr in an apparent dose-dependent manner. Plasma zinc depression was accompanied by an increase in hepatic MT content as well as MT-associated total Zn and ^{65}Zn used to pulse-label the metalloprotein. The depression in plasma zinc, but not iron, and the enhanced synthesis of MT was significantly reduced by prior treatment of rats with actinomycin D. This finding suggests a requirement for new mRNA synthesis for zinc, but not iron alterations during hypersensitivity reactions. Results support the concept that induction of hepatic MT may be a common mechanism involved in altered plasma zinc homeostasis regardless of the initiating pathophysiologic condition.

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TABLE I. EFFECT OF HYPERSENSITIVITY REACTIONS ON CONCENTRATIONS OF PLASMA ZINC AND IRON AND ZINC ASSOCIATED WITH HEPATIC METALLOTHIONEIN (MT).

Group (n)	Treatment[a]		Plasma[b]		Liver MT[b,c]	
	Sensitization (mg BSA)	Challenge (mg BSA)	Zn (µg/dl)	Fe (µg/dl)	Zn (µg/dl)	^{65}Zn (cpm/ml)
1 (10)	5.0	0.5	73 ± 5[d]	123 ± 10[d]	82 ± 7[d,e]	1351 ± 132[d,e]
2 (10)	5.0	None	125 ± 4	195 ± 10	18 ± 3	216 ± 32[f]
3 (10)	None	0.5	114 ± 6	159 ± 6	20 ± 4	310 ± 45

a Rats were challenged 14 days after initial sensitizing dose of BSA. All measurements were made 7 hr after challenge. See Methods section for details.

b Values represent means ± SEM.

c Values expressed in terms of concentration found in metallothioneins isolated from specified volume of heat-treated (85°) hepatic cytosol (5).

d Significantly different, $P \leq 0.001$, vs. group 2 or 3.

e n = 9

f n = 8

TABLE II. EFFECT OF ACTINOMYCIN D PRETREATMENT ON THE DEPRESSION IN PLASMA ZINC AND IRON AND ENHANCED HEPATIC METALLOTHIONEIN (MT) CONTENT INDUCED BY HYPERSENSITIVITY REACTIONS.

Group (n)	Treatment[a]		Plasma[b]		Liver MT[b]	
	Sensitization (mg BSA)	Actinomycin D pretreatment (+) and challenge (mg BSA)	Zn (µg/dl)	Fe (µg/dl)	Zn[c] (µg/dl)	MT[d] (mg)
1 (10)	10	+, 0.5	136 ± 5[e]	90 ± 7[f]	87 ± 8[e,f,g]	89 ± 9[e,f,g]
2 (10)	10	-, 0.5	45 ± 4[f]	93 ± 5[f]	197 ± 17[f]	160 ± 10[f]
3 (10)	None	-, 0.5	125 ± 3	166 ± 9	23 ± 2	17 ± 2

a Rats were challenged 14 days after sensitizing dose of BSA. See methods section for other details of experimental protocol.

b Values represent means ± SEM.

c Values expressed in terms of concentration found in metallothioneins isolated from specified volume of heat-treated (85°) hepatic cytosol (5). Each ml of cytosol represents approximately 0.3 g of liver.

d Values determined gravimetrically by densitometric analysis of stained protein bands separated by SDS-PAGE and expressed as mg/peak area for combined A and B forms of MT. Equivalent amounts of liver cytosol on a wet-liver weight basis were applied to gels.

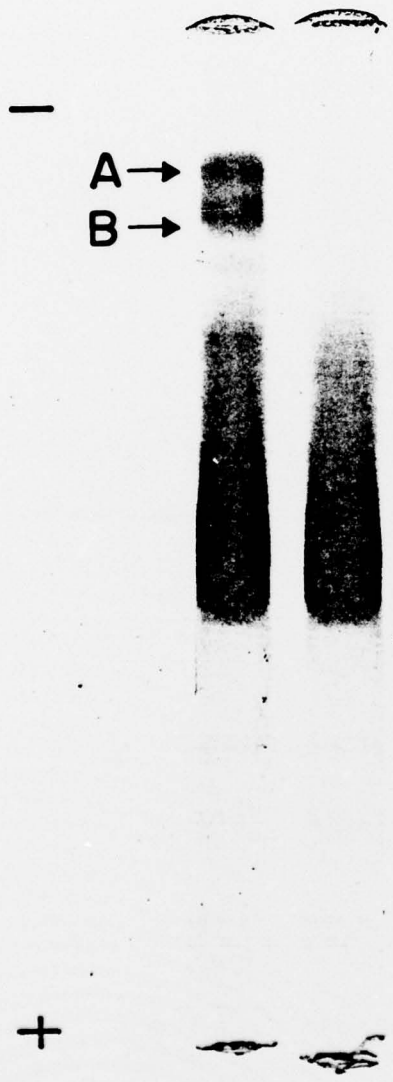
e Significantly different, $P \leq 0.001$, vs. group 2.

f Significantly different, $P \leq 0.001$, vs. group 3.

g n = 9

LEGENDS TO FIGURES

Figure 1. Typical separation by SDS-gel electrophoresis of A and B forms of metallothioneins isolated from livers of BSA-sensitized rats with (left) and without (right) antigen challenge. Migration was towards anode for 1.5 h at ambient temperature and constant current (2.5 mA/gel). Amount of material applied to each gel was obtained from equivalent quantities of hepatic cytosol (see Ref. 5). Material migrating ahead of metallothioneins has not been identified.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